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I, KAY WARD, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 2337 for a patent by MINISTER OF AGRICULTURE, MINISTER FOR LAND AND WATER CONSERVATION FOR AND ON BEHALF OF THE STATE OF NEW SOUTH WALES filed on 19 August 1999.

WITNESS my hand this
Twenty-ninth day of August 2000

K Ward

KAY WARD
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ORIGINAL

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: Recombinant Sub-Unit Vaccine

The invention is described in the following statement:

RECOMBINANT SUB-UNIT VACCINE

Field of the Invention

The present invention relates generally to the field of therapeutics and the development thereof for use in animals including mammals, humans, birds and fish. More particularly, it relates to sub-unit vaccines that are effective against pathogens causing infections thereof for use in animals including mammals, humans, birds and fish.

Background Art

Scientific background

The development of therapeutics and in particular vaccines directed against pathogens such as viruses, bacteria, protozoans, fungi is ongoing. Such research has proved invaluable in preventing the spread of disease in animals including humans. In fact, in modern medicine, immunotherapy including vaccination has eradicated smallpox and virtually eradicated diseases such as polio, tetanus, tuberculosis, chicken pox, and measles.

Generally, ideal vaccines have a long shelf life, are capable of inducing with a single dose long lasting immunity against a pre-selected pathogen and all of the phenotypic variants, are incapable of causing the disease to which the vaccine is directed against, are effective therapeutically and prophylactically, are easily prepared using economical standard methodologies and can be administered easily in the field.

There are four major classes of commercially available vaccines. They include non-living whole organism vaccines, live attenuated vaccines, vector vaccines, and sub-unit vaccines. Vaccination with non-live materials such as proteins generally leads to an antibody response or CD4+ helper T cell response while, vaccination with live materials (eg infectious viruses) generally leads to a CD8+ cytotoxic T-lymphocyte (CTL) response. A CTL response is crucial for protection against pathogens like infectious viruses and bacteria. This poses a practical problem, for the only certain way to achieve a CTL response is to use live agents that are themselves pathogenic. The problem is generally circumvented by using attenuated viral and bacterial strains or by killing whole cells that can be used for vaccination. These strategies have worked well but the use of attenuated strains always carries the risk that the attenuated agent may recombine genetically in the host and turn into a virulent strain. Thus, there is need for therapeutics and methods that can lead to CD8+ CTL response by vaccination with non-live materials such as proteins in a specific manner.

Sub-unit vaccines have provided one means for dealing with some of these problems. Such vaccines generally comprise a sub-cellular component derived from a pathogen of interest. A sub-unit component can be either produced from a defined sub-cellular fraction of the pathogen, be a purified protein, nucleic acid or a polysaccharide. All of these elements have an antigenic determinant capable of stimulating an immune response against the pathogen of interest. Generally, the sub-cellular component of the sub-unit vaccine is obtained either by purifying a preparation of disrupted pathogen or synthesised using well-known procedures.

There are, however, several limitations associated with sub-unit vaccines. First, a requirement for the production of such a vaccine is that the antigenic determinant(s) must be characterised and identified. This imposes limitations on their use, particularly against highly variable antigenic determinants. Second, sub-unit vaccines are generally ineffective in stimulating cytotoxic T cell responses. Third, the immunity conferred by sub-unit vaccines is often short lived and therefore requires continual booster injections. Very few recombinant expressed sub-unit vaccines have been shown to induce strong and long lasting immunity in vaccinated animals (including man). One notable exception is the recombinant surface antigen Hepatitis B vaccine used in man. One of the problems associated with the use of such vaccines appears to be in correctly presenting the antigens to the immune system such that strong humoral (antibody) immunity and strong cell mediated (via T cells) immunity are induced. In particular, existing recombinant (sub-unit) vaccines do not appear to result in strong 'memory' responses such that vaccinated animals react very quickly when they are exposed to natural infections caused by a pathogen.

By way of example only, deficiencies in current sub-unit vaccines prepared from pestiviruses like bovine viral diarrhoea virus (BVDV) have been extensively reported on. These studies have shown that even though large amounts of recombinant protein were used in the vaccines, there were poor protection rates seen showing that the vaccines failed to protect from challenge with live BVDV isolates (either homologous protection or heterologous protection).

The present invention seeks to provide an improved therapeutic vaccine which ameliorates at least some of the disadvantages over existing prior art.

General background

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively, and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

Bibliographic details of the publications referred to in this specification are collected at the end of the description. All references cited are hereby incorporated by reference. No admission is made that any of the references constitute prior art.

Throughout this specification unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Summary of the Invention

The present invention generally relates to complexes of insect cell derived heat shock proteins (hsps) coupled to at least a heterologous antigenic molecule, which when administered to an animal is capable of eliciting an immunological response in the host. Desirably, the complex is capable of enhancing the animal's immunocompetence against a pathogen.

Thus, in one embodiment the present invention provides an improved sub-unit vaccine capable of inducing an immune response in an animal comprising: an insect cell hsp coupled to an antigenic heterologous peptide or polypeptide. Preferably, the insect cell hsps are non-covalently coupled to the antigenic peptide or polypeptide(s).

The present invention also provides methods for preparing an insect cell hsp – antigenic heterologous peptide or polypeptide complex comprising: (a) introducing into an insect cell a nucleotide sequence encoding at least a antigenic peptide or polypeptide(s), said nucleotide sequence being introduced into the cell in such a manner that translation of the nucleotide sequence is possible when the sequence is within the insect cell; (b) culturing the cell under conditions that provide for expression of the peptide or polypeptide; (c) exposing the cell to a stress that is capable of initiating the production of heat shock proteins in that cell; and (b) recovering the expressed complex. This procedure can also be accompanied by the step of: purifying the complex by any means known in the art. In a preferred embodiment, the complex produced by the method is isolated from insect cell polypetides.

Pharmaceutical compositions produced according to the invention comprise an insect cell heat shock protein coupled to an antigenic heterologous peptide or polypeptide as described herein together with a pharmaceutically acceptable carrier and are useful in therapeutic methods for inducing an immune response against the antigenic heterologous peptide or polypeptide.

In a further embodiment the invention provides a method for inducing immunocompetence in a animal against a pathogen, said method comprising the steps of: administering to an animal a therapeutically effective amount of an insect cell hsp coupled to an antigenic peptide or polypeptide and a pharmaceutically acceptable carrier.

Preferably the methods of the invention comprise methods of eliciting an immune response in an individual in whom the treatment or prevention of infectious diseases is desired by administering a composition comprising a therapeutically effective amount of a complex, in which the complex consists essentially of hsps non-covalently bound to an antigenic molecule using any convenient mode of administration. A variety of administrative techniques may be utilized, among them oral administration, nasal and other forms of transmucosal administration, parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like.

DETAILED DISCLOSURE OF THE INVENTION

Insect cell hsps coupled to at least an antigenic peptide/polypeptide provide an alternative therapeutic vaccine to those discussed in the background art, for stimulating an animal's immune system to elicit an immune response against foreign pathogens. While hsps have been included in therapeutic formulations, no one has, to the best of the applicant's knowledge, employed hsps from a heterologous class or even species or animal hsps, and more particularly insect cell hsps, coupled to at least an antigenic peptide/polypeptide.

Collective features which different sub-unit vaccines produced according to the present invention display include:

(1) The complex is completely non-infectious.

(2) The complex is safe for use in animals since baculoviruses do not infect animal cells.

(3) Therapeutics produced according to the invention will be cheaper to manufacture in that much higher yields of antigenic proteins can be produced from baculovirus-infected insect-cell cultures than from comparable systems.

(4) Therapeutics developed according to the invention have been found to generate very strong memory responses in animals. Thus

when an animal is subsequently challenged with a pathogen they mount a very rapid and strong response to that pathogen.

Thus, in one embodiment the present invention provides an improved sub-unit vaccine capable of inducing an immune response in an animal comprising: an insect cell hsps coupled to an antigenic heterologous peptide or polypeptide. Preferably, the insect cell hsps are non-covalently coupled to the antigenic peptide or polypeptide(s).

Heat shock proteins (hsps) are synthesized by a cell in response to heat shock. The major hsps can accumulate to very high levels in stressed cells, but they occur at low to moderate levels in cells that are not stressed. Insect heat shock proteins, useful in the practice of the instant invention are proteins whose intracellular concentration increases when an insect cell is exposed to a stressful stimuli, they are capable of binding other proteins or peptides, and are capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH.

The phrase "insect cell hsp(s)–antigenic peptide/polypeptide complex", as used herein, refers to any complex that can be isolated from insect cell culture that comprises insect cell hsps coupled to at least a heterologous peptide or polypeptide having at least one antigenic determinant. Preferably, the coupling is achieved using non-covalent bonding.

The term "heterologous peptide or polypeptide", as used herein, refers to a peptide or polypeptide not naturally located in an insect cell. Preferably it is something not normally endogenously complexed with insect hsps *in vivo* and does not normally co-purify with insect hsps.

The term “polypeptide” as used herein, refers to any amino acid sequence longer than a peptide and which may or may not be modified by chemical means. Such a sequence would include dipeptides, oligopeptides, proteins and the like including modified forms thereof.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule.

Antigenic molecules can be selected from among those known in the art or selected by their ability to bind to antibody or MHC molecules or generate immune responses. They include any molecule that will induce an immune response against the infectious agent, e.g., antigens of viruses, bacteria, fungi, parasites etc. In a preferred embodiment of the invention the antigenic molecules may be derived from, but are not limited to: (1) viral proteins such as, proteins of any of the immunodeficiency viruses including human immunodeficiency virus type I (HIV-I) and human immunodeficiency virus type II (HIV-II), flaviviruses, pestiviruses like bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV), hepatitis type A, hepatitis type B, hepatitis type C, hepatitis type E, hetatitis type G (GB), influenza, Varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, hantavirus, coxsackie virus, mumps virus, measles virus, rubella virus and polio virus; (2) antigenic bacterial proteins selected from, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria and legionella; (3) antigenic protozoa proteins selected from, but not limited to, leishmania, coccidia, and trypanosoma; and (4) anitgenic parasite proteins selected from, but not limited to, chlamydia and rickettsia.

The phrase "immune response" refers to any cellular process that is produced in the animal following stimulation with an antigen and is directed toward the elimination of the antigen from the animal. The immune response typically is mediated by one or more populations of cells characterized as being lymphocytic and/or phagocytic in nature.

The term “vaccine” as used herein, refers to mean any composition containing an insect hsp coupled to a peptide or polypeptide having at least one antigenic determinant which when administered to a animal is capable of stimulating an immune response against the antigenic determinant. It will be understood that the term vaccine does not necessarily imply that the composition will provide a complete protective response. Rather a therapeutic effect will be sufficient.

The term "adjuvant" as used herein, refers to a compound or mixture that enhances the immune response to a composition containing an insect hsp coupled to a peptide or polypeptide having at least one antigenic determinant. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response. Adjuvants include, but are not limited to, Quil A, Isocomatrix adjuvant, saponin adjuvant complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet haemocyanins and dinitrophenol. Preferably, the adjuvant is pharmaceutically acceptable.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the *U.S. Pharmacopeia* or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or soluble saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

The phrase "therapeutically effective amount" as used herein refers to an amount sufficient to stimulate by at least about 15%, preferably by at least 50%, more preferably by at least 90%, and most preferably completely, a animals immune system causing it to generate an immunological memory against the antigenic determinant.

The term “mammal” as used herein refers to a class of vertebrates whose young feed upon milk from the mother's breast. Most species (except cetaceans) are more or less hairy, all have a diaphragm, and all (except the monotremes) are viviparous. The term will be understood to include for example human, farm animals (i.e., cattle, horses, goats, sheep and pigs), household pets (i.e., cats and dogs) and the like.

The term “animal” as used herein refers to animal species including, but not limited to, birds, fish and mammals.

The term "cytotoxic T cell", as used herein, refers to any T lymphocyte expressing the cell surface glycoprotein marker CD8+ that is capable of targeting and lysing a target cell which bears a major histocompatibility class I (MHC Class I) complex on its cell surface and is infected with an intracellular pathogen.

The term "cytokine" refers to any secreted polypeptide that influences the function of other cells mediating an immune response. Some examples of cytokines include, but are not limited to, interleukin-1.alpha. (IL-1.alpha.), interleukin-1.beta. (IL-1.beta.), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon .alpha. (IFN.alpha.), interferon .beta. (IFN.beta.), interferon .gamma. (IFN.gamma.), tumor necrosis factor .alpha. (TNF.varies.), tumor necrosis factor .beta. (TNF.beta.), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor .beta. (TGF-.beta.).

The immune response generated against an introduced insect cell hsps–antigenic peptide or polypeptide complex will be dictated by the amino acid constitution of the antigenic determinants located on the peptide or polypeptide in the complex. Such determinants may define either humoral or cell mediated antigenic regions. Without being limited to any particular mode of action, it is contemplated that the immune response generated by the insect cell hsps-antigenic peptide or protein complex will preferably include both humoral and cell mediated immune responses. Where a cell mediated immune response is effected it preferably leads to a T cell cascade, and more specifically by means of a cytotoxic T cell cascade. The term "cytotoxic T cell", as used herein, refers to any T lymphocyte expressing the cell surface glycoprotein marker CD8+ that is capable of targeting and lysing a target cell which bears a class I histocompatibility complex on its cell surface and is infected with an intracellular pathogen.

Diseases that might be treated or prevented by the methods of the present invention are caused by pathogens including, but not limited to viruses, bacteria, fungi protozoa and parasites.

Viral diseases that can be treated or prevented by the methods of the present invention include, but are not limited to, those caused by immunodeficiency viruses including human immunodeficiency virus type I (HIV-I) and human immunodeficiency virus type II (HIV-II), flaviviruses, hepatitis type A, hepatitis type B, hepatitis type C, pestiviruses like bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV), influenza, Varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, hantavirus, coxsackie virus, mumps virus, measles virus, rubella virus and polio virus.

Bacterial diseases that can be treated or prevented by the methods of the present invention are caused by bacteria including, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria and legionella.

Protozoal diseases that can be treated or prevented by the methods of the present invention are caused by protozoa including, but not limited to, leishmania, coccidia, and trypanosoma.

Parasitic diseases that can be treated or prevented by the methods of the present invention are caused by parasites including, but not limited to, chlamydia and rickettsia.

While the present invention may be prepared by various means, preferably it is made *in vitro* using insect cells.

Thus the present invention also provides methods for preparing an insect cell hsp – antigenic heterologous peptide or polypeptide complex comprising: (a) introducing into an insect cell a nucleotide sequence encoding at least a antigenic peptide or polypeptide(s), said nucleotide sequence being introduced into the cell in such a manner that translation of the nucleotide sequence is possible when the sequence is within the insect cell; (b) culturing the cell under conditions that provide for expression of the peptide or polypeptide; (c) exposing the cell to a stress that is capable of initiating the production of heat shock proteins in that cell; and (b) recovering the expressed complex. This procedure can also be accompanied by the step of: purifying the complex by any means known in the art. In a preferred embodiment, the complex produced by the method is isolated from insect cell polypetides.

While it will be appreciated that there are various ways to introduce a nucleotide sequence into an insect cell in a manner that permits translation of the sequence, preferably the nucleotide is inserted into a cloning vector designed for expressing peptides or proteins in insect cells. Such vectors will be known to those in the field. Desirably the nucleotide sequence encoding the antigenic peptide or polypeptides is inserted into a recombinant baculovirus that has been genetically engineered to produce antigenic peptide or polypeptides, for instance, by following the methods of Smith *et al* (1983) Mol Cell Biol 12: 2156-2165.

A wide variety of insect cells may also be used in the present invention. Preferably the insect cells are derived from a Lepidopteran species, eg *Spodoptera frugiperda* such as the Sf9 and SF21cell lines.

By way of example only coupling of an antigenic proteins to insect-cell hsps *in vitro* may be accomplished quite simply by placing the cells (in a container) in a water bath at, for example, 43^{0}C for approximately 10 mins to heat shock the cells. The cells are then incubated at about 27.5^{0}C for a further 24 hrs to allow expression of coupled recombinant protein and hsps. At the end of 72 hrs, harvesting of the recombinant-protein cultures is carried out.

In another embodiment, the present invention provides methods for enhancing a animal's immunocompetence and the activity of its immune effector cells against a pathogen. Such methods will include the step of: administering a composition comprising a therapeutically effective amount of an insect cell hsp–antigenic peptide/polypeptide complex, in which the complex consists essentially of a hsp coupled to an heterologous peptide or polypeptide antigenic molecule.

In a highly preferred embodiment, the present invention provides hsps complexes prepared from proteins and polypeptides derived from bovine viral diarrhoea virus (BVDV).

According to this embodiment there is provided a sub-unit therapeutic against BVDV infections in cattle herds that is capable of inducing an immune response in cattle comprising: an insect cell hsp coupled to an antigenic BVDV peptide or polypeptide. Preferably, the insect cell hsps are non-covalently coupled to the antigenic BVDV peptide or polypeptide(s).

The primary aim of all modern pestivirus therapeutics is based on their ability to prevent the transplacental transmission of the virus thus breaking the cycle of infection in cattle herds. The foetal protection index is the only objective measurement of efficacy of BVDV vaccines. It has increasingly been adopted overseas as the demonstrated requirement for future BVDV vaccine registrations.

A surprising feature of this embodiment of the invention is that 100% protection has been observed using the vaccine prior to BVDV infections in cattle. Such protection has resulted from the unusually high levels of neutralising antibodies to BVDV after only a short exposure to the live virus challenge. The sub-unit vaccine provides levels of neutralising antibodies never observed before by the applicants. In addition, the sub-unit vaccine provides a surprisingly effective memory cytotoxic T cell response. Further, the sub-unit vaccine provides for the first time a 100% effective inhibition of transplacental transfer of virus from dam to foetus.

In addition to the above, vaccines produced in accordance with this embodiment of the invention have particular advantages over other BVDV vaccines and in particular BVDV sub-unit vaccines. One of the drawbacks of using live attenuated vaccines or whole organism vaccines is the likelihood of infection of cell cultures during manufacture of the vaccine. In contrast, sub-unit vaccines are non-infectious. In particular, the sub-unit vaccine of the invention does not require serum for manufacture, thus alleviating the risks involved with handling serum products such as foetal calf serum.

Another advantage of these vaccines is their safety when vaccinating commercial herds of animals such as cattle against BVDV. The sub-unit vaccine of the present invention can be used safely in animals without the risk of infection (live attenuated viruses) or infection of the animal cells with the virus. Thus it is safe to use in all animals, including pregnant animals.

A further advantage of BVDV sub-unit vaccines produced in accordance with the invention is the cost-effective nature of producing the sub-unit vaccine. It is possible to obtain high yield of antigenic proteins. In particular, in a preferred embodiment of the invention a sub-unit vaccine is produced using a vector baculovirus to infect insect cell cultures, producing a sub-unit vaccine effective against BVDV. The resulting vaccine has efficacy against a much wider range of antigenically diverse BVDV isolates.

Any antigenic region from BVDV may be used in the identified sub-unit vaccines. Preferably, the antigenic peptide or polypeptides are derived from the major immunogenic regions E0, E1/E2 and NS3. In a highly preferred form of this embodiment of the invention the sub-unit vaccine is produced using a truncated NS3 protein from isolates of BVDV. Surprisingly, this NS3 protein antigen does not cause the production of a detectable range of antibodies in the serum of cattle vaccinated with the sub-unit vaccine. Thus the incorporation of the NS3 ~~protein into the sub-unit vaccine provides a useful marker to distinguish infected~~

cattle within a herd from vaccinated cattle. It is the preferred practice in Europe and the US to include a marker in the vaccine, identifying infected animals from vaccinated animals. Thus the sub-unit vaccine of the present invention provides an excellent marker for distinguishing infected animals within a herd from vaccinated animals.

Pharmaceutical Compositions

The invention provides pharmaceutical compositions comprising an insect cell hsp–antigenic peptide or polypeptide complex that enhances the immunocompetence of the host individual and elicits specific immunity against pathogens. The therapeutic regimens and pharmaceutical compositions of the invention are described below. These compositions are believed to have the capacity to prevent the onset and progression of infectious diseases.

In general, comprehended by the invention are pharmaceutical compositions comprising therapeutically effective amounts of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions may include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present complexes. *See, e.g.*, *Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

Pharmaceutical compositions may be for administration by injection, or prepared for oral, pulmonary, nasal or other forms of administration. The mode of administration of the complexes prepared in accordance with the invention will necessarily depend upon such factors as the stability of the complex under physiological conditions, the intensity of the immune response required, the type of pathogen etc.

Preferably, the complex is administered using standard procedures, for example, intravenously, subcutaneously, intramuscularly, intraorbitally, ophthalmically, intraventricularly, intracranially, intracapsularly, intraspinally, intracisternally, intraperitoneally, buccal, rectally, vaginally, intranasally, orally or by aerosol administration.

Parenteral Delivery

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers and administered by any parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections. In addition the formulations may optionally contain one or more adjuvants.

Oral Delivery

Contemplated for use herein are oral solid dosage forms, which are described generally in *Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatised with various polymers (*E.g.*, U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in *Modern Pharmaceutics*, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the formulation will include the insect cell hsp–antigenic peptide/polypeptide complex (or a chemically modified form thereof), and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above derivatised insect cell hsp–antigenic peptide/polypeptide complexes. In this respect the complexes may be chemically modified so that oral delivery is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the protein (or peptide) molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the protein and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Abuchowski *et al.*, 1981, *supra*; Newmark *et al.*, *J. Appl. Biochem.*, 4:185-189 (1982). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the insect cell hsp–antigenic peptide/polypeptide complex the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. One skilled in the art has available formulations that will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the complex or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance, a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic *i.e.* powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the insect cell hsp–antigenic peptide/polypeptide complex may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, alpha-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulphate, magnesium lauryl sulphate, polyethylene glycol of various molecular weights, and Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the complex during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulphate, dioctyl sodium sulphosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the complex either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the complex are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The complex could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms *i.e.*, gums. Slowly degenerating matrices may also be incorporated into the formulation. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e.

the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film-coated tablet; the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Pulmonary Delivery

Also contemplated herein is pulmonary delivery of the complex. The insect cell hsp–antigenic peptide/polypeptide complex may be delivered to the lungs of an animal while inhaling and traverses across the lung epithelial lining to the blood-stream.

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered-dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of the complex. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified protein may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the complex suspended in water at a concentration of about 0.1 to 25 mg of biologically active protein per ml of solution. The formulation may also include a buffer and a simple sugar (*e.g.*, for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the complex suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the complex and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, *e.g.*, 50 to 90% by weight of the formulation. The protein (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 microns, most preferably 0.5 to 5 microns, for most effective delivery to the distal lung.

Nasal Delivery

Nasal delivery of the complex is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

Administration with other compounds

The therapeutic regimens and pharmaceutical compositions of the invention may be coadministered with additional immune response enhancers or biological response modifiers including, but not limited to, the cytokines IFN-.alpha., IFN-.gamma., IL-2, IL-4, IL-6, TNF, or other cytokine affecting immune cells. In accordance with this aspect of the invention, the complexes of the hsp and antigenic molecule are administered in combination therapy with a therapeutically active amount of one or more of these cytokines. As used herein, the term "cytokine" is meant to mean any secreted polypeptide that influences the function of other cells mediating an immune response. Accordingly, it is contemplated that the complex can be coadministered with a cytokine to enhance the immune response directed against the tumor. Preferred cytokines include, but are not limited to, interleukin-1.alpha. (IL-1.alpha.), interleukin-1.beta. (IL-1.beta.), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon .alpha. (IFN.alpha.), interferon .beta. (IFN.beta.), interferon .gamma. (IFN.gamma.), tumor necrosis factor .alpha. (TNF.varies.), tumor necrosis factor .beta. (TNF.beta.), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor .beta. (TGF-.beta.).

In addition, conventional antibiotics may be coadministered with the stress protein-peptide complex. The choice of suitable antibiotics will however be dependent upon the disease in question.

Dosages

For all of the above molecules, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain the proper dosage.

Typically, the complex should be administered in an amount sufficient to initiate in the animal an immune response against the pathogen following subsequent challenge. The amount of insect cell hsp–antigenic peptide/polypeptide complex administered preferably is in the range of about 0.1-1.0 micrograms of complex/kg body weight of the animal/administration, and most preferably about 0.2 to 0.5 micrograms of complex/kg body weight of the animal/administration.

It is contemplated that a typical dose will be in the range of about 0.5 to about 50 micrograms for a human subject weighing about 75 kg. In addition, it is contemplated that the strength of the immune response may be enhanced by repeatedly administering the complex to the individual. Thus in one example the animal may receive at least two doses of the insect cell hsp–antigenic peptide/polypeptide complex at approximately monthly intervals. If necessary, the immune response may be boosted at a later date by subsequent administration of the complex. It is contemplated, however, that the optimal dosage and immunization regimen may be found by routine experimentation by one skilled in the art.

Kits

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the insect cell hsp–antigenic peptide/polypeptide complex in pharmaceutically acceptable form. The hsp-antigenic molecule complex in a vial of a kit of the invention may be in the form of a pharmaceutically acceptable solution, e.g., in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of hsp-antigenic molecule complexes by a clinician or by the patient.

BEST MODE(S) FOR CARRYING OUT THE INVENTION

Further features of the present invention are more fully described in the following non-limiting Figures and Examples. It is to be understood, however, that this description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above. In the drawings:

Figure 1. The development of anti-E2 (neutralising) antibody concentration in both vaccinated (-o-) and control (-■-) groups of heifers before and after live virus challenge (C).

Figure 2. The development of anti-NS3 antibody concentration in both the vaccinated (-o-) and control (-■-) groups of heifers before and after virus challenge (C).

Methods of molecular cloning, and protein chemistry methods that are not explicitly described in the following Examples are reported in the literature and are known by those skilled in the art. General texts that described conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook *et al*. (1989); Glover (1985); and Ausubel, *et al. Current protocols in molecular biology.*

Example 1

Production of recombinant baculoviruses and expression of recombinant pestivirus proteins

Pestiviruses

Pestivirus isolates Trangie-D10, Bega and Clover Lane were isolated and characterised at the Virology Department, Elizabeth Macquarie Agricultural Institute (EMAI). The BVDV isolates Trangie D10 and Bega represent Bovine Viral Diarrhoea Virus (BVDV) type 1 pestiviruses, while the Clover Lane isolate is a Border Disease Virus (BDV) isolate.

The GenBank accession numbers of the Australian virus isolates used in the sub-unit vaccine and the relative positions of the genomic fragments used relative to the reference strain BVDV NADL (Accession number M31182) were as follows: Bega (AF049221) E0 partial sequence codes relative to the whole virus genome from 1171-1897; Trangie (AF049222) E0 partial sequence codes relative to the whole-virus genome from 1171-1897; Bega (AF049225) E1 and E2 partial sequence codes relative to the whole-virus genome, from 2253-3490; Trangie (AF049223) E1 and E2 partial sequence codes relative to the whole-virus genome, from 2290-3490; Clover Lane (AF037405, Becher *et al.*, 1998) E1 and E2 partial sequence codes relative to the whole virus genome, from 2360-3510; Bega (AF052303) NS3, NS4a partial sequence codes relative to the whole-virus genome, from 5416-7591; Trangie (AF052304) NS3, NS4a partial sequence codes relative to the whole-virus genome, from 5675-7528.

Extraction of viral RNA

cDNA was transcribed from Australian BVDV isolates for all of the major immunogenic regions (E0, E1/E2 and NS3) using standard techniques. Briefly, viral RNA was extracted from infected cells and/or viral pellets using either RNAzol (Biotex Laboratories, Inc) or TRIzol® Reagent (Gibco BRL), according to the manufacturer's instructions. Dried RNA pellets were reconstituted in 10 µl or 20µl sterile Diethyl pyrocarbonate (DEPC) (Sigma) treated water (Sambrook *et al.*, 1989).

Reverse transcription to produce cDNA for E1/E2, NS3 and E0 immunogenic regions

cDNA was produced for E1/E2 by reverse transcription by preparing an E1/E2 reverse transcriptase (RT) mixture as described in Table 1. Tubes were heated in an FTS-960 Thermal Sequencer (Corbett Research) at 37°C for 50 mins, followed by 70°C for 10 mins to denature the reverse transcriptase. The RT mix was cooled at 5°C for 2 mins prior to cDNA amplification by polymerase chain reaction (PCR).

cDNA for NS3 was also prepared by RT by preparing a NS3 RT mixture according to Table 2. Tubes were heated in an FTS-960 Thermal Sequencer at 37°C for 59 mins, followed by 94°C for 15 mins to denature the reverse transcriptase prior to cDNA amplification by PCR.

cDNA for E0 was produced by preparing a RT mixture as described in Table 3. Tubes were heated in an FTS-960 Thermal Sequencer at 37°C for 50 mins, followed by 70°C for 10 mins to denature the reverse transcriptase. The RT mix was then cooled at 5°C for 2 mins prior to cDNA amplification by PCR.

Table 1

Reagent	**Volume**	**Final concentration**	**Supplier**
X10 PCR buffer [100 mM Tris-HCl; 15 mM $MgCl_2$; 500 mM KCl; pH 8.3]	2.0 µL	X1 [10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; pH 8.3]	Boehringer Mannheim
25 mM $MgCl_2$	2.8µL	3.5mM	Sigma molecular biology grade
dinucleotide triphosphate (dNTP) containing 5 mM each dATP, dGTP, dCTP and dTTP	4µl	1 mM of each dNTP	Boehringer Mannheim
random hexamers (50 µM in 10 mM Tris-HCl, pH 8.3;	1 µl	2.5 µM	Perkin Elmer
RNase-inhibitor	10 units	-	Boehringer Mannheim
M-MLV	12.5 units	-	Gibco BRL
RNA preparation	1µl	-	-
	20 µl	-	-

Table 2

Reagent	Volume	Final concentration	Supplier
X5 first strand buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$)	4 μL	X1 (50 mM Tris-HCl, pH 8.3; 55 mM KCl; 3 mM $MgCl_2$:)	Gibco BRL
0.1 M DTT (dithiothreitol	2 μL	0.01m	Gibco BRL
dinucleotide triphosphate (dNTP) solution (containing 5 mM each dATP, dGTP, dCTP and dTTP),	2 μL	0.5 Mm	Boehringer Mannheim
random hexamers (50 μM in 10 mM Tris-HCl, pH 8.3)	1 μL	2.5 μM	Perkin Elmer
RNase-inhibitor	20 units	-	(Boehringer Mannheim
Superscript™ II (RNase H^- Reverse Transcriptase)	50 or 100 units	-	Gibco BRL
RNA preparation Heated to denature at 65°C for 5 mins and cooled rapidly on ice before use	1μL	-	-
Total volume	20 μL	-	-

Table 3

Reagent	Volume	Final concentration	Supplier
X10 PCR buffer [100 mM Tris-HCl; 15 mM $MgCl_2$; 500 mM KCl; pH 8.3]	2.0 μL	X1 [10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; pH 8.3]	Boehringer Mannheim
25 mM $MgCl_2$	2.8μL	3.5mM	Sigma molecular biology grade
dinucleotide triphosphate (dNTP) containing 5 mM each dATP, dGTP, dCTP and dTTP	4μl	1 mM of each dNTP	Boehringer Mannheim
random hexamers (50 μM in 10 mM Tris-HCl, pH 8.3;	1 μl	2.5 μM	Perkin Elmer
RNase-inhibitor	10 units	-	Boehringer Mannheim
M-MLV	12.5 units	-	Gibco BRL
RNA preparation	1μl	-	-
	20 μl	-	-

PCR oligonucleotide primers

PCR primers for the BVDV isolates, Trangie and Bega, were based on conserved regions of the published sequences for overseas pestivirus isolates. The primers for the BDV isolate, Clover Lane, were made using its published sequence (Becher *et al.,* 1998). Primers were designed using the computer programme 'Primer Designer - Version 2.0' (Scientific and Educational Software, 1990, 1991) and contained restriction sites incorporated to enable directional cloning of the cDNA (Tables 4 and 5).

Amplification of cDNA by Polymerase Chain Reaction

The amplification of cDNA from E1/E2, NS3 and E0 followed a similar procedure. The amplification reaction was carried out in a total volume of 100µl. To the 20µl of RT, 8 ml x10 PCR buffer (100 mM Tris-HCl; 15 mM $MgCl_2$; 500 mM KCl; pH 8.3: Boehringer Mannheim), 7.2 ml 25 mM $MgCl_2$ (to give a final concentration of 3.3 mM $MgCl_2$; Sigma, molecular biology grade), 2.5 units *Taq* DNA polymerase (Boehringer Mannheim) and 1 µl each of the sense and antisense primers (30 pmol per ml) were added.

E1/E2 cDNA was initially denatured at 95°C for 2 mins, followed by 35 cycles of denaturation at 95°C for 30 secs, annealing at 55°C for 30 secs and

Table 4

Pestivirus Protein	Primer sequence[a]	Location of primer in NADL sequence[h]
Trangie E1/E2[b*]	5'-CGC**GGATCC**AGTGCTGGCATTTGAAGA-3' Bam HI	2290
Bega E1/E2[c*]	5'-CGC**GGATCC**CAGACTGGTGGCCTTATG-3' Bam HI	2253
CloverLane E1/E2[d*]	5'-CAC**GGATCC**AGTGCATCAACAACAGCCT-3' Bam HI	2360
Trangie E0[e◆]	5'-CGC**GGATCC**AGTTTTGTTTCAAGTTACAATG-3' BamHI	1171
Bega E0[f◆]	5'-CGC**GGATCC**AGTTTTGTTTCAAGTTACAATG-3' BamHI	1171
Trangie NS3[g♣]	5'-AA**CTGCAG**ACTAGAGTGGTTTGCCAAAGCAACA-3' Pst I	5675

[a] Restriction enzyme sites are shown in bold, [b] GenBank accession number is AF049223, [c] GenBank accession number is AF04925, [d] GenBank accession number is AF037405 Becher *et al.* (1998), [e] GenBank accession number is F049222, [f] GenBank accession number is Af049221, [g] GenBank accession number is AF052304, [h] GenBank accession number is M31182 Collett *et al.* (1988), [*]E1/E2 fragments code for a protein containing 69 amino acids (aa) from E1 and finishing 35 aa before the end of E2, [◆] codes for the full length E0 protein, [♣] codes for NS3 protein without the serine protease enzyme and includes the area coding for a T-cell epitope found in CSFV (Pauly *et al.*, 1995).

Table 5

Pestivirus Protein	Primer sequence[a,b]	Location of primer in NADL sequence[i]
Trangie E1/E2[c*]	5'-GCG**AAGCTT**AGGACTCTGCGAAGTAATC-3' Hind III Stop	3490
Bega E1/E2[d*]	5'-CATG**CCATGG**TTAGGACTCTGCGAAGTAATC-3' NcoI Stop	3490
CloverLane E1/E2[e*]	5'-CGC**AAGCTT**ACGCTACCACTGCCAACATGA-3' Hind III Stop	3510
Trangie E0[f♦]	5'-CGC**AAGCTT**AGACATCACAGTAAGGGGA-3' Hind III Stop	1897
Bega E0[g♦]	5'-CGC**AAGCTT**AGACATCACAGTAAGGGGA-3' Hind III Stop	1897
Trangie NS3[h✤]	5'-ACGT**CCATGG**TTAAGCTTGATAGCCTACGTACC-3' Nco I Stop	7528

[a]Restriction enzyme sites are shown in bold, [b]In frame stop codon is underlined in the anti-sense primer, [c]GenBank accession number is AF049223, [d]GenBank accession number is AF 049225, [e]GenBank accession number is AF037405 Becher et al. (1998), [f]GenBank accession number is AF049222, [g]GenBank accession number is AF049221, [h]GenBank accession number is AF052304, [i]GenBank accession number is M31182 Collett et al. (1988), *E1/E2 fragments code for a protein containing 69 amino acids (aa) from E1 and finishing 35 aa before the end of E2, ♦ codes for the full length E0 protein, ✤ codes for NS3 protein without the serine protesase and includes the area coding for T-cell epitope found in CSFV (Pauly et al., (1995).

extension at 72°C for 1 min. A final extension step of 72°C for 5 mins was included, before cooling the tubes to 5°C for 2 mins.

The Clover Lane (BDV) PCR mix did not require $MgCl_2$ and only 1unit of *Taq* DNA polymerase was needed for amplification.

E0 amplification was carried out as described for E1/E2, with the exception that the initial denaturing step was at 94°C for 2 mins.

Amplification of NS3 cDNA was carried out in a total volume of 50 μl using the total 20 μl from the reverse transcription reaction, to which was added 3μl x10 PCR buffer (100 mM Tris-HCl; 15 mM $MgCl_2$; 500 mM KCl; pH 8.3: Boehringer Mannheim), 2 μl 25 mM $MgCl_2$ (to give a final concentration of 3 mM $MgCl_2$; Sigma, molecular biology grade), 1-2 units *Taq* DNA polymerase (Boehringer Mannheim) and 1 μl each of the sense and antisense primers (25-30 pmol per ml). An initial denaturing step at 94°C for 3 min was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min. A final extension step of 72°C for 5 min was included, before cooling the tubes to 4°C.

Cloning of PCR fragments

PCR products were purified with PCR SPINCLEAN™ columns (Progen Industries, Limited), according to the manufacturer's instructions. If the PCR reaction produced non-specific bands in addition to the required product, or subcloning from another plasmid was necessary, the DNA was further purified by elution from a 0.8% agarose gel, using a modification of the method described by Heery (1990).

Purified PCR fragments were digested and ligated into pBlueBacHis A, B or C baculovirus transfer vectors (MaxBac Baculovirus Expression System, Invitrogen Corporation) containing compatible cohesive overhangs, using standard cloning protocols (Sambrook *et al.*, 1989; Current Protocols in Molecular Biology, 1991). A, B or C vectors provide three different reading frames to achieve protein expression in the baculovirus expression system (Table 6).

Table 6

Pestivirus Protein	pBlueBacHis A, B or C transfer vector
Trangie E1/E2	C
Bega E1/E2	C
Clover Lane E1/E2	A
Trangie E0	B
Bega E0	B
Trangie NS3	B

NS3 proved difficult to clone directly into the pBlueBacHis baculovirus transfer vector and was thus first cloned into pCR™II plasmid (Invitrogen Corporation) using the Invitrogen TA Cloning Kit. The methods for this procedure were carried out according to the manufacturer's instructions. The NS3 fragment was then sub-cloned into the pBlueBacHis B vector as described for the other fragments of the genomes.

Transformation of baculovirus plasmids with the PCR fragments

The ligations were transformed into competent *E. coli* strain Top 10 (Invitrogen Corporation), Genotype: F$^-$*mcr*A D(*mrr-hsd*RMS-*mcr*BC) f80*lac*ZDM15 D*lac*X74 *deo*R *rec*A1 *ara*D139 D(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G, and/or Sure® *E. coli* (Stratagene), Genotype : e14$^-$(McrA$^-$)D (*mcrCB-hsdSMR-mrr*)_171 *endA1 supE44 thi-1 gyrA96 rel A1 lac recB recJ sbcc umuc::*Tn5 (kan^r) *uurC[F' proAB lacI*^{a}*Z* D m15 Tn*10*(Tet^r)]^c. Protocols for the preparation of competent cells and transformation of the bacteria were taken from the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8.

Screening bacterial clones for plasmid containing PCR fragment and plasmid purification for transfection

Bacterial clones containing pBlueBacHis + PCR fragment were identified by growing colonies, extracting the plasmids using the boiling miniprep method described in Sambrook, *et. al.* (1989), and then undertaking restriction digests of the plasmids to verify those containing the correct-sized insert. Recombinant plasmids were purified to a level suitable for transfection reactions using plasmid purification kits (QIAGEN Pty Ltd., tip-20 or tip-100 columns), according to the manufacturer's instructions.

Production of purified recombinant baculoviruses by Cationic liposome transfection of Sf9 cells to produce recombinant baculoviruses

Recombinant baculoviruses were produced by co-transfecting linearised wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA and baculovirus transfer vector containing PCR fragment into Sf9 cells, by the technique of cationic liposome mediated transfection. This was carried out according to the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8. Some minor modifications were made in relation to volumes, but these were not significant in terms of the overall strategy used.

Plaque purifying recombinant baculoviruses

Recombinant virus was plaque purified three times before virus master stocks were prepared, ensuring the virus was cloned from a single particle and no wild-type virus was present. Plaque assays were set up according the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8.

After each round of plaque purification, the recombinant viruses were screened using a modified Pestivirus antigen-capture ELISA (PACE) (Shannon *et al.*, 1991). The modified method involved supernatant + cells (50 µl/well) being added directly to a blocked, washed ELISA plate, and the plate incubated for 1 hr at 37^{O}C. Antibody solution (50 µl/well) was then added. The antibody used was either biotinylated goat anti-pestivirus antiserum or individual anti-E2 or anti-NS3 monoclonal antibodies (mAbs). The plate was incubated overnight at 22^{O}C, then developed as described by Shannon *et al.* (1991), omitting the incubation with biotinylated anti-mouse IgG for samples that were reacted with the biotinylated goat antiserum. It should be noted that recombinant, baculovirus-expressed E0 has not been detected in the PACE.

Recombinant baculovirus master, seed and working stocks

The master virus stock for each of the recombinant baculoviruses constructed was made according the Invitrogen MaxBac Baculovirus Expression System Manual Version 1.8. The titre of the stock was determined by a plaque assay, as described above, except that the cells were overlaid with 1.5% carboxymethylcellulose (CMC, BDH; 6% CMC in deionised water, diluted 1 in 4 with complete TC100 + X-gal [125µg/ml, Boehringer Mannheim]). After 7 days, the blue plaques were counted to give the virus titre.

The seed and working stock were made from the master and seed stock, respectively using a low MOI of 0.1 to 0.5pfu/ml. All virus stocks were stored at

4°C for use in vaccine production. For long term storage of Master, Seed and Working stocks, each recombinant virus was ampouled and frozen at -80°C.

Optimisation of recombinant protein production

Sf9 insect-cell suspensions, adapted to Sf-900 II Serum Free Media according to the protocol described by Gibco BRL (1995), were used to optimise recombinant protein expression. Two conical flasks, containing 50ml cells (1.5×10^6 cells per ml), were infected with recombinant baculovirus at a high and low MOI, between 0.1 and 5.0. A third flask acted as an uninfected control culture. The 3 flasks were incubated with shaking at 28°C, and 5ml aliquots removed at 24 hr intervals for up to 7 days.

The samples were centrifuged at room temperature (RT) for 10 min at 900 x *g*, and the supernatants carefully removed. The pellets and supernatants were stored at -20°C until daily sampling was completed. The amount of specific, recombinant pestivirus protein in the samples was then determined using the modified PACE described above. The cell pellets were reconstituted in 200µl or 250µl NP-40 (1% [v/v] in PBS), vortexed and centrifuged at RT for 10 min at 900 x *g*. Serial dilutions of the pellet extract (in 1% [v/v] NP40) were assayed. The culture supernatants were assayed undiluted, as well as serially diluted (in 1% [v/v] NP40).

It was found that the cell viability was reduced at the higher rate of infection. Therefore, an MOI or 0.1 to 2 was more appropriate. Data for the optimised expression times and the recombinant protein location in the insect-cell suspension cultures, either supernatant or pelleted-cell fraction are shown in Table 7.

In relation to the expressed E2 proteins, the hydrophobic 'tail' region of this protein was deliberately omitted when constructing the cDNA of the genome encoding this region and therefore in preparing the recombinant baculoviruses responsible for protein expression in the insect-cell cultures. This resulted in the

Table 7

Recombinant protein	Time to harvest after infection (Hours)	Recombinant protein location in infected cultures
Trangie E1/E2	48	Supernatant
Bega E1/E2	48	Supernatant
Clover Lane E1/E2	48	Supernatant
Bega E0	48 [a]	Supernatant + cells[b]
Trangie E0	48 [a]	Supernatant + cells[b]
Trangie NS3	48	Cells only

[a] These proteins could not be detected in the PACE, therefore the time to harvest was based on the times at which the other recombinant proteins were harvested.

[b] Both supernatant and cells need to be harvested since the location in the insect cell suspension cultures could not be accurately determined.

majority of these proteins being transported from the insect cells via normal protein-export pathways and secretion into the cell culture medium. Therefore, maximum protein recovery was from the supernatant fraction of the insect-cell cultures.

In contrast, the expressed NS3 protein remains 'bound' within the insect cells and therefore were harvested from pelleted cells at the end of the culture time. Cells were pelleted on a bench centrifuge at 2000 x *g* for 10 mins, which maximised the harvest of this recombinant protein in a small volume. In the case of the E0 (E^{rns}) recombinant, expressed proteins, the location of these could not be determined and, therefore, culture supernatant plus insect cells were harvested.

Example 2

Coupling of immunogenetic BVDV proteins to insect cell hsps *in vitro*.

The insect cells used throughout the example were *Spodoptera frugiperda* 9 (Sf9) cells (Invitrogen Corporation, USA).

Establishing Sf9 insect cells in monolayers

This method was based on the Invitrogen MaxBac Baculovirus Expression System Manual, Version 1.8. Frozen cell aliquots (normally 1.0ml vials stored in a liquid Nitrogen tank) were thawed rapidly at 37°C, the vial wiped with 70% (v/v) ethanol and the cells transferred to a 75 cm^2 tissue culture flask containing 15 ml complete TC100 medium (Gibco BRL TC100, supplemented with 10% FCS [Trace Biosciences Pty Ltd] and containing penicillin and streptomycin [100 units/ml each]) at room temperature (RT).

After the flask had been incubated for 1 hr at 28°C (Clayson Incubator, Edwards Instrument Company) to allow the cells to attach, the medium was replaced with 20 ml fresh, complete TC100. The medium was changed every 48 hrs until the cells were confluent, at which stage they were passaged as follows. The medium was removed and replaced with 5 ml fresh medium, the cells gently scraped off the bottom of the flask, and 1 ml of cell suspension was transferred to a new 75 cm^2 tissue culture flask containing 19 ml of complete TC100 at RT.

After rocking the flask to distribute the cells evenly across the bottom, the flask was placed at 28^{O}C and the cells grown as described above.

Culture of Sf9 insect cells in suspension

This method was based on the Invitrogen MaxBac Baculovirus Expression System Manual, Version 1.8. Cells were grown as suspension cultures after they had been passaged 3-4 times as a monolayer culture. The medium used for these cultures was complete TC100 containing 1% (v/v) pluronic F-68 (10% solution; Gibco BRL). To produce the suspension cultures, medium was first removed from the cells which were a confluent monolayer and immediately replaced with 5 ml of fresh medium. The cells were gently scraped from the bottom of the flask and the cell suspension transferred to a 100 ml, screw-topped, conical flask containing 25 ml of medium containing pluronic. The flask was incubated at 28^{O}C, shaking on an orbital shaker (Bio-Line; Edwards Instrument Company) for at least 1hr at 80 rpm before increasing the shaking speed to 110 rpm for the remainder of the growth period. When the cell density had reached approximately 2x10^{6} cells/ml, an additional 30 ml of medium was added. Cells were then grown on in 50 ml volumes and passaged when the cell density reached approximately 2x10^{6} cells/ml. At this time, cells were diluted in medium to give approximately 0.5x10^{6} cells/ml, and incubated at 28^{O}C, shaking at 110 rpm, until passaged again.

Adaptation of Sf9 insect cells to grow in suspension culture in serum-free medium

Insect cells were adapted to grow in suspension culture in serum-free medium according to the protocol described by Gibco BRL (1995). Cells that had been growing in complete TC100 with pluronic were transferred to 50 ml of serum-free medium (Sf-900 II SFM; Gibco BRL) containing penicillin and streptomycin (100 units/ml each) at a density of 5x10^{5} cells/ml. The cells were grown on to a density of 2x10^{6} cells/ml, then passaged at a density of 5x10^{5} cells/ml. This was continued until a cell density of 2x10^{6} cells/ml, with a cell viability of at least 80%, was reached. The cells were then considered to be adapted to grow in serum-free medium, and subsequently were passaged using serum-free medium in the same way as described for cells grown in complete TC100 medium with pluronic F-68, as described for the culturing of Sf9 insect cells in suspension.

Requirements for Sf9 cells used to express recombinant pestivirus proteins

Sf9 cells in serum-free medium, used for the expression of recombinant pestivirus proteins, were required to be below 30 passages in culture. Conical flasks (500ml) were seeded with Sf9 cells at a density 0.5×10^6 cells/ml in a final volume of 150ml. When the cells in these flasks reached a density of 1.0 - 1.5×10^6 cells/ml, they were infected with the appropriate recombinant baculovirus encoding the required, expressed pestivirus protein.

Production of the recombinant baculoviruses encoding pestivirus proteins

Production of recombinant baculoviruses and expression of recombinant pestivirus proteins is described in Example 1.

Multiplicity of Infection

The Sf9 insect cells were infected at a multiplicity of infection (MOI) ranging between 0.1 and 2.0, depending on the individual recombinant-baculovirus stock titre. Results are shown in Table 8.

Induction of heat shock proteins in cultures of Sf9 cells infected with recombinant baculoviruses

The experimental times and temperatures used to induce the production of heat shock proteins in insect-cell cultures were optimised for Sf9 insect-cell cultures expressing specific pestivirus recombinant proteins, as set out below.

Heat shock conditions for NS3 recombinant baculovirus-infected cultures

Sf9 insect cells infected with the recombinant baculovirus expressing the truncated pestivirus NS3 protein were incubated at 28°C, with shaking at 110 rpm, for 48 hours. The infected cell cultures flasks were then placed in a 43°C water bath along with a similar 'dummy' cell culture flask containing a thermometer in 150ml of water. A 10 min incubation was started when the

Table 8

Recombinant Baculovirus	EMAI Virus number	Stock	Titre (pfu/ml)	Multiplicity of Infection
AcMNPV+ Trangie E1/E2	Z044	Master	2.8×10^7	1.0
AcMNPV+ Bega E1/E2	Z376	Seed	1.0×10^7	1.0
AcMNPV+ Clover lane E1/E2	Z361	Working	4.35×10^7	2.0
AcMNPV+ Bega E0	Z341	Master	3.2×10^6	0.2
AcMNPV+ Trangie E0	Z293	Seed	2.2×10^7	1.0
AcMNPV+ Trangie NS3	Z346	Master	2.0×10^6	0.2

thermometer reached 43^{0}C, and then every 2 mins, the flasks were given a gentle mix by swirling the medium and cells within the flask. This was determined to be the optimal heat-shock conditions for Sf9 cells expressing pestivirus recombinant proteins. The cell culture flasks were then placed back into the incubator (at 28^{O}C), with shaking at 110 rpm, for a further 2-hr period to allow the insect-cell, heat-shock proteins (hsps) to be expressed and coupled to the pestivirus NS3 recombinant protein.

Heat shock conditions for E1/E2 and E0 recombinant baculovirus-infected cultures

Sf9 insect cells infected with recombinant baculoviruses expressing either E1/E2 or E0 pestivirus proteins were incubated at 28^{O}C, with shaking at 110 rpm, for a period of 24 hr. The infected cell cultures were then heat shocked exactly as described above (10 min at 43^{O}C). The cell cultures in their flasks were then returned to the incubator (28^{O}C) and the cells cultured, with shaking at 110 rpm, for a further period of 24 hr. In this system, the cultures expressing the E1/E2 proteins were heat shocked at 24hr, as opposed to 48hr for the NS3 protein, to ensure that the E1/E2 recombinant proteins were coupled to insect cell hsps prior to their transport out of the Sf9 cells and into the cell culture medium. Since it had not yet been determined whether the recombinant E0 pestivirus proteins were secreted from insect cells, or remained within the cells themselves, Sf9 cultures producing the E0 pestivirus proteins were also heat shocked at 24 hr after infection with the recombinant baculoviruses.

Heat shock conditions for uninfected, control-cell cultures

Uninfected Sf9 insect cell cultures were incubated at 28^{O}C (shaking at 110 rpm) for a period of 48 hr. These control cell cultures were heat shocked exactly as described above. The cell culture flasks were then returned to 28^{O}C in the incubator, with shaking at 110 rpm, for a further 2hr incubation period to allow the insect cell heat shock proteins to be formed by the stressed cells.

Harvesting of individual recombinant pestivirus proteins from Sf9 cell cultures [NS3 recombinant protein + heat-shock proteins (hsps)]

Cells were separated from the medium by centrifugation at 2000 x *g* for 10 mins. The cell pellet, containing the NS3 antigen, was then resuspended in one sixth of the original volume using serum-free medium (Sf-900 II SFM; Gibco BRL) containing leupeptin (protease inhibitor, ICN Biomedicals, Inc) at a concentration of 5μg/ml. This gave an effective six-fold concentration of the cells plus recombinant NS3 antigen. The cells were then freeze/thawed twice at -80^{0}C to break down cellular membranes and release the NS3 recombinant protein, together with recombinant baculovirus, into the medium.

Harvesting of individual recombinant pestivirus proteins from Sf9 cell cultures [E1/E2 recombinant protein + heat-shock proteins (hsps)]

Cells were removed from the medium by centrifugation at 2000 x *g* for 10 mins. Leupeptin (protease inhibitor) was again added to the supernatant, containing the expressed E1/E2 protein, to give a final concentration of 5μg/ml. The addition of the protease inhibitor prevented degradation of the expressed proteins.

Harvesting of individual recombinant pestivirus proteins from Sf9 cell cultures [E0 recombinant protein +heat-shock proteins (hsps)]

In the case of insect-cell cultures expressing this particular protein, the whole culture (cells plus medium) was harvested and leupeptin added to give a final concentration of 5μg/ml. The culture was then freeze/thawed twice to break down cellular membranes, releasing both the recombinant baculoviruses and any cell-associated E0 proteins into the medium.

Harvesting of individual recombinant pestivirus proteins from Sf9 cell cultures [Control cells + heat-shock proteins (hsps)]

Control (uninfected) cultures were harvested as described above. However, the control cells were concentrated 10-fold.

Beta-Propiolactone (βPL) Inactivation of Recombinant Baculoviruses

βPL inactivation (using β-propiolactone, Sigma Aldrich Fine Chemicals) was carried out twice on all recombinant protein preparations produced by the baculovirus-vector expression-vector system, and for the control-cell preparation. The standard method employed by the Commonwealth Serum Laboratories (CSL, "Inactivation of Baculovirus using Beta-Propiolactone, 1998") was used in all cases. To ensure no residual infectious baculovirus was left in the "inactivated" material, each preparation was passaged three times in Sf9 monolayers. The final pass was titrated in an Sf9 plaque assay, using 1.5% carboxymethylcellulose (CMC, BDH; 6% CMC in deionised water diluted, 1 in 4 with complete TC100) containing 125mg/ml X-gal (Boehringer Mannheim) as the overlay. Plaque assays were set up according the Invitrogen MaxBac Baculovirus Expression System Manual, Version 1.8, and the plaque assay read on day 7. There was no evidence of live, infectious baculovirus present in any of the preparations used to formulate the sub-unit vaccine, thus meeting the Australian Quarantine Inspection Service (AQIS) requirements for the use of the experimental vaccine in food-producing animals.

Concentration of the Recombinant E0 and E1/E2 Protein Preparations.

The Sf9 cells expressing the E0 recombinant proteins were separated from the medium after inactivation (as above) by centrifugation at 2000 x *g* for 10 min and these cells were then stored at 4°C pending their use. The supernatants containing recombinant E0 proteins were then concentrated five times in separate Amicon Ultrafiltration Cell steps, according to the manufacturer's instructions. The concentrated E0 protein-containing supernatant was then re-mixed with the E0 Sf9 cells to prepare the final, concentrated preparation.

In the case of the inactivated E1/E2 recombinant proteins, the supernatant fractions only were concentrated using the Amicon Ultrafiltration Cell. These proteins are all secreted from the recombinant-baculovirus infected cells and therefore the cell fraction is discarded.

Determination of the amount of recombinant pestivirus protein in each preparation by titration in the Pestivirus antigen capture ELISA (PACE).

The amount of recombinant protein, after βPL inactivation, was assayed by titrating each individual recombinant protein preparation in the modified PACE (see Shannon *et al.*, 1991). The modification of the published method involved sample (50 µl/well) being added directly to a blocked, washed ELISA plate, and the plate incubated for 1 hr at 37°C. Antibody solution (50 µl/well) was then added. The antibody used was either biotinylated goat anti-pestivirus antiserum (pAb) or individual anti-E2 or anti-NS3 monoclonal antibodies (mAbs). The plate was incubated overnight at 22°C, then developed as described in Shannon *et al.* (1991), omitting the incubation with biotinylated anti-mouse IgG for samples that were reacted with the biotinylated goat antiserum.

It should be noted that recombinant E0 protein is not able to be detected in this assay system since the protein failed to react with either of the polyclonal or monoclonal antibodies. Therefore, it was assumed that this protein was similar in concentration to those determined for the analogous E1/E2 expressed structural glycoproteins.

Summary of the recombinant proteins incorporated in the sub-unit vaccine

The recombinant pestivirus proteins Trangie NS3, Trangie E0, Bega E0, Trangie E1/E2, Bega E1/E2, Clover Lane E1/E2, together with the Control cells, were prepared by the methods described in this example. However, Bega and Trangie E1/E2 were not heat shocked, Trangie E1/E2 was concentrated six times instead of five and the BDV Clover Lane E1/E2 recombinant protein was processed as described for the recombinant E0 protein preparations.

Recombinant, experimental sub-unit vaccine

The composition of the vaccine preparations used in Example 4 are set out in Table 9. In summary, each dose of the recombinant pestivirus vaccine contained: 1ml Bega E0, 1ml of Trangie E0, 1ml of Clover Lane E1/E2, 0.5ml Trangie E1/E2, 1ml of Bega E1/E2 and 0.3ml of Trangie NS3. Thimerosal

Table 9

Summary of Sub-unit, expressed - protein Vaccine preparation	
Trangie NS3	Labelled : TNS3 8/4/98 MOI 0.2, grown for 48hrs in SFM Heat shocked at 43°C for 10 mins Placed back in incubator for 2hrs with shaking ONLY cells harvested (S/N discarded), therefore [ ] 6X Leupeptin added to give 5ug/ml Freeze/thawed 2 times BPL inactivated 2 times using CSL standard method Stored at -80°C (Block 5)
Trangie E0	Labelled : TE0 23/4/98 MOI 1, grown for 48hrs in SFM Heat shocked at 43°C for 10 mins Placed back in incubator for 24hrs with shaking Cells + S/N harvested Leupeptin added to give 5ug/ml Freeze/thawed 2X BPL inactivated 2 times using CSL standard method AMICON concn. 5X Stored at -80°C (block 5)
Bega E0	Labelled : BE0 23/4/98 MOI 0.2, grown for 48hrs in SFM Heat shocked at 43°C for 10 mins Placed back in incubator for 24hrs with shaking Cells + S/N harvested Leupeptin added to give 5ug/ml Freeze/thawed 2X BPL inactivated 2 times using CSL standard method AMICON concn 5X Stored at -80°C (Block 5)
Clover Lane E1/E2	Labelled : CLE2 23/4/98 MOI 2, grown for 48hrs in SFM

	Heat shocked at 43°C for 10 mins Placed back in incubator for 24hrs with shaking Cells + S/N harvested Leupeptin added to give 5ug/ml Freeze/thawed 2 times BPL inactivated 2 times using CSL standard method AMICON concn. 5X Stored at -80°C (Block 5)
Trangie E1/E2	Labelled : TE2 8/5/98 MOI 1, grown for 48hrs in SFM ONLY S/N harvested Leupeptin added to give 5ug/ml Freeze/thawed 2 times BPL inactivated 2 times using CSL's standard method AMICON concn. 6X Stored at -80°C (Block 5)
Bega E1/E2	Labelled : BE2 8/5/98 MOI 1, grown for 48hrs in SFM ONLY S/N harvested Leupeptin added to give 5ug/ml Freeze/thawed 2 times BPL inactivated 2 times using CSL standard method AMICON concn. 5X Stored at -80°C (Block 5)
Control Vaccine	~~Labelled : SFM-Sf9 24/6/98~~ Grown for 48hrs in SFM Heat shocked at 43°C for 10 mins Placed back in incubator for 24hrs with shaking Cells harvested and taken up in 50ml SFM therefore [ ]10X Leupeptin added to give 5ug/ml Freeze/thawed 2 times BPL inactivated once using CSL standard method Stored at -80°C (Block 5)

Table 9 (continued)

Mixing of recombinant proteins to produce the sub-unit vaccine

Rec Antigen	1 x DOSE	27 x DOSES
BEO	1ml	27ml
TE0	1ml	27ml
CLE1/E2	1ml	27ml
TE1/E2	0.5ml	13.5ml
TNS3	0.3ml	8.1ml
BE1/E2	1ml	27ml
Total Volume		129.6ml

Added 1.29ml Thimerosal to 129.6ml vaccine mix, took out 9.6ml and placed into 1ml aliquots for storage at -20°C (freezer in Block 5 egg room).

To the remaining 120ml, added 32.5ml Iscomatrix adjuvant and stirred for 2 mins to mix well.

Aliquoted into 2 containers i.e 68ml/container and stored at 4°C.

Set up vaccine in 10ml syringes with 18 gauge needles - 6ml/dose final volume.

Control Vaccine

	1 dose	6 doses
Control cell preparation	4.8ml	28.8ml
Iscomatrix adjuvant	1.3ml	7.8ml
Thimerosal	0.048ml	0.29ml

Set up control vaccine in 10ml syringes with 18 gauge needles - 6ml/dose final volume (mercuric compound, Sigma Aldrich) was added to the vaccine mixture to help prevent bacterial contamination, with a final concentration in the vaccine of 0.1%(w/v) . Isocomatrix adjuvant (Commonwealth Serum Laboratories, Australia) was used at the rate of 2mg incorporated in each vaccine dose. The formulated vaccine was stored at 4°C until injected into the cattle (initial dose followed by a sec dose 4 weeks later).

Control Vaccine

The formulation of the Control vaccine is also set out in Table 9. In summary, each dose of control vaccine contained 4.8ml of the control-cell preparation. Thimerosal was again added to the control vaccine to help prevent bacterial contamination, the final concentration in the vaccine being 0.1%(w/v). Isocomatrix adjuvant (CSL) was incorporated at 2mg per vaccine dose, in line with the rate used in the experimental vaccine. The control vaccine preparation was stored at 4°C until required. Two doses were given to the control animals in the trial on the same days as the experimental sub-unit vaccine was administered to the vaccinated animals.

Example 3

The effect of the sub-unit vaccine on Australian cattle

Format of the sub-unit foetal protection trial

A total of 22 pestivirus antibody negative, non-pregnant heifers were selected for the trial. A group of animals (n=10) were vaccinated twice, 4 weeks apart, with the sub-unit protein vaccine (6 ml) as prepared in examples 1 and 2. A further group of animals (n=12) were vaccinated with the control preparation (6 ml). All animals were bled at regular intervals and the concentrations of both anti-E2 and anti-NS3 antibodies were determined using the complex-trapping-blocking ELISA (CTB-ELISA) format as carried out by the Elizabeth Macarthur Agricultural Institute (EMAI).

Immediately after the second vaccination, the animals were synchronised for oestrus. Insemination occurred immediately after oestrus was detected. All animals were judged to have become pregnant and have developing foetuses of greater than 6 weeks of age, at 11 weeks after the second vaccination, a time considered to be the most susceptible for infection with the challenged BVDV isolate. The heifers were then challenged with a dose (3×10^6 $TCID_{50}$) of the live heterologous BVDV isolate Glen Innes.

Six weeks following viral challenge, all heifers were slaughtered at an export abattoir (Mudgee) in the two groups. The foetuses were collected from pregnant heifers. That is, there were 7 foetuses from the 10 animals in the vaccinated group and there were 9 foetuses from the 12 animals in the control group.

Individual foetal tissues were collected under sterile conditions. Several methods were employed to test for the presence of BVDV infection. Firstly, two antigen-capture ELISAs specific for either E2 antigens or NS3 antigens were used. Secondly, a panel of monoclonal antibodies was used to detect infected cells isolated using standard techniques and immunoperoxidase (IPX) staining. Thirdly, a 5'-UTR virus-specific RT-PCR was used. The combination of these methods gave a sensitive and specific detection of infected versus non-infected foetuses collected from the heifers. Preliminary results are shown in Table 10 and Table 11.

Table 10

Dilution	TNS3 + HSP	S/N ratio	BNS3 (control no hsp)
1/25	0.316	2.75	0.115
1/50	0.423	2.00	0.211
1/100	0.097	0.46	0.114
1/200	0.262	2.60	0.101
1/400	0.126	1.26	0.100
1/800	0.106	0.80	0.128

Table 11

Antigen Dilution	TNS3 + HSP	S/N ratio	BNS3 (control, no hsp)
0	0.321	4.94	0.065
1/10	0.323	1.74	0.186
1/20	0.326	1.35	0.241
1/40	0.324	1.34	0.241
1/80	.0325	1.80	0.180
1/160	.0301	1.40	0.209

Effect of E2 sub-unit vaccine on cattle immune response to BVDV

The average concentration of anti-E2 (neutralising) antibody in both the vaccinated and control groups of heifers, before and after vaccination, and after live virus challenge, is shown in Figure 1.

The average concentration of anti-E2 antibody plotted over time indicated that the sub-unit vaccine resulted in very high concentrations of anti-E2 antibody in the vaccinated group. High titers of antibody commenced as early as 2 weeks after the administration of the second dose of vaccine. The concentration of anti-E2 antibody in vaccinated heifers was significantly higher than in the control group. The concentration of anit-E2 in the vaccinated group declined slightly over the preceding 9 weeks, but still remained significantly higher than the control group of heifers.

A rapid anamnestic rise in the concentration of E2 antibody in the vaccinated group was observed at 7 days post challenge with the live BVD virus, which continued to rise until 9 days post challenge, where it remained at a sustainable maximum concentration. In contrast to this trend, an increase in the concentration of anti-E2 antibody was only observed in the control group after challenge with the live virus. The onset of a normal response in the control group was then observed, with the average concentration of anti-E2 antibody beginning to develop at 14 days post challenge. However, a maximum response was not reached until 3 to 4 weeks post challenge.

Thus the vaccination of pregnant heifers with the sub-unit vaccine creates an immune response in the heifer during the first 4 to 7 days after viral infection. This is an important stage during which the live virus crosses the placenta to the developing fetus. These results clearly indicate that the replication of the live virus was antagonised in the sub-unit vaccinated group of heifers (n=10). This is the first time that such a response has been reported for a sub-unit vaccine.

Effect of NS3 sub-unit vaccine on cattle immune response to BVDV

The concentration of anti-NS3 antibody in both vaccinated and control groups of heifers over time is shown in Figure 2.

Surprisingly, there was no anti-NS3 antibody detected in the vaccinated heifers after vaccination. The reason for this is not yet known. However, this result has a great potential for the development of a "marker" vaccine. All "naturally infected" animals develop anti-NS3 antibodies 21 days after infection with BVDV. Since animals vaccinated with the sub-unit vaccine do not develop anti-NS3 antibodies (discussed below), they are easily distinguishable from "naturally infected" animals.

It is likely that anti-NS3 protein results in the generation of a strong cell-mediated immune response through the induction of CD8+ cytotoxic T cells although failing to elicit an antibody response.

After challenge with the live virus, vaccinated heifers (7 out of 10) showed no significant development of anti-NS3 antibodies until 5 to 6 weeks post challenge. The remaining three vaccinated heifers developed anti-NS3 antibodies 3 to 6 weeks post challenge. However, the concentration of antibodies was significantly lower than the control group of heifers. In contrast, the control heifers (n=12) developed a normal antibody response commencing 14 to 18 days post challenge, reaching a peak 4 weeks post challenge (Figure 2).

These results clearly indicate that the replication of the live virus was inhibited in the sub-unit vaccinated group of heifers (n=10). This is the first time that such a response has been reported for a sub-unit vaccine. It is evident that the postulated early onset of CTL responses directed against infected cells prevented the replication of the virus. Thus there was insufficient virus circulating in the vaccinated animals to cross the placenta and infect the fetus.

The concentration of neutralising antibodies induced by the sub-unit vaccine

Serum neutralisation tests (SNTs) were carried out using different BVDV isolates to investigate the concentration of neutralising antibodies induced by the sub-unit vaccine, and to determine the anamnestic responses resulting from live virus challenge.

The results of this experiment are shown in Table 12. As expected from the results shown in Figure 1, no antibody response was observed prior to

Vaccinated Animal Number	SNT at 4 weeks post second vaccination with subunit vaccine[✦]					SNT at 7 days post challenge with Glen Innes virus[✣]			SNT at 14 days post challenge with Glen Innes virus[✣]		
	Trangie	Bega	Clover Lane	Glen Innes	Braid-wood	Trangie	Bega	Clover Lane	Trangie	Bega	Clover Lane
Q239	>2056	>2056	32	>2056	1024	12800	12800	800	128000	64000	8000
Q269	512	128	16	256	64	3200	3200	~4	16000	16000	64
Q300	256	512	128	512	128	6400	12800	800	64000	32000	8000
Q306	1028	256	16	1028	256	12800	51200	1600	64000	64000	4000
Q324	1028	512	128	1028	256	3200	1600	400	64000	16000	4000
Q354	128	64	<4	128	16	3200	1600	100	64000	64000	2000
Q355	1028	>2056	32	2056	512	12800	12800	1600	512000	256000	16000
Q379	2056	>2056	32	>2056	1024	25600	12800	1600	128000	64000	8000
Q382	512	512	64	>2056	64	6400	6400	100	256000	256000	8000
R346	>2056	>2056	64	>2056	1024	51200	51200	3200	512000	256000	64000
Mean Titre (Range)	**1028** (128-2056)	**1020** (64->2056)	**50** (0-128)	**1200** (128->2056)	**436** (16-1024)	**13700** (3200-51200)	**16600** (1600-51200)	**1024** (~4-3200)	**180000** 16000-512000)	**108000** (16000-256000)	**12250** (64-64000)

(✦) = serum dilutions started at 1 in 4; 2-fold dilutions to end-point.;
(✣)= serum dilutions started at 1 in 1000 for Trangie and Bega, 1 in 100 for Clover Lane; 2-fold dilutions to end-point

vaccination in the vaccinated group of heifers. However, after the second vaccination, a very good anti-E2 neutralising antibody response (average titre of 1 in 1000) was observed in the 2 BVDV isolates associated with the vaccine (Trangie and Bega). In contrast, there was a very low response of neutralising antibody (average titre of 1 in 50) against the sheep BDV isolate (Clover Lane) even though the recombinant E2 protein from this virus was incorporated in the vaccine in combination with hsps. However, the resulting concentration of neutralising antibody was greater from this sub-unit vaccine than that achieved with the use of inactivated whole Clover Lane virus in a previous experiment (results not shown).

SNTs conducted on the heterologous challenge virus Glen Innes indicated a surprisingly high cross-reactivity (average titre 1 in 1200) at 4 weeks after the second dose of vaccine. This finding confirms that the combination of E2 proteins results in good cross protection against heterologous viruses. An even more distant BVD virus Braidwood showed a lower concentration of neutralising antibodies (average titre of 1 in 400) but does correspond with a significant antibody production against infection with this virus.

The SNT assays carried out on serum collected from the vaccinated animals at 7 and 14 days post challenge (Table 13) were even more surprising. Assays were conducted using each of the 3 viruses represented in the sub-unit vaccine. It is evident from the results that there was an extremely high anamnestic response in the anti-E2 antibody levels at just 7 days post challenge. Average SNT for both Trangie and Bega BVDV viruses were in the order of 1 in 14 000 to 16 000 at 7 days but rose to an extraordinary concentration by day 14 post challenge. At 14 days post challenge, the average titre against Trangie was 1 in 180 000, with 2 animals having titres as high as 1 in 512 000. Similarly, titres against Bega were on average 1 in 100 000 at 14 days, with 3 animals having titres against Bega of 1 in 256 000. The magnitude of these titres is rarely seen in "naturally infected" animals.

Table 13

Vaccinated Animal Number	**Reciprocal of SNT (NPLA) Titre (4 weeks after second vaccination in both trials)**					
	Inactivated Vaccine 1112/96 (T+B+CL)			**Sub-unit Vaccine 584/98 (T+B+CL)**		
	Trangie✦	**Glen Innes***	**Clover Lane✦**	**Trangie✦**	**Glen Innes***	**Clover Lane✦**
1	1024	512	10	>2048	>2048	32
2	256	512	20	512	256	16
3	256	512	20	256	512	128
4	40	64	10	1024	1024	16
5	50	50	10	1024	1024	128
6	200	256	8	128	256	<4 (0)
7	-	-	-	1024	2048	32
8	-	-	-	2048	>2048	32
9	-	-	-	512	>2048	64
10	-	-	-	>2048	>2048	64
Mean Titre **(Range)**	**300** (40-1024)	**300** (50-512)	**12** (8-20)	**1028** (128-2048)	**1200** (128->2048)	**50** (0-128)

Comparison of the neutralising titres against 3 viruses measured in cattle at 4 weeks after vaccination with either the inactivated, whole-virus vaccine or the non-infections, sub-unit vaccine containing recombinant proteins. (✦) Trangie (BVDV isolate) and Clover Lane (BDV isolate) incorporated in both vaccines. In sub-unit vaccine, Clover Lane E2 recombinant protein was coupled *in vivo* with heat shock proteins (hsps); (*)= Glenn Innes was the challenge live virus used in both trials (a BVDV isolate clearly distinct from the vaccine viruses).

Thus it can be concluded that the sub-unit vaccine had a significant effect in "priming" animals for a reaction against live virus challenge that has not previously been observed.

The SNT assays indicated a strong "priming" response against the BDV isolate Clover Lane (Table 12). At 7 days post challenge with a totally unrelated live virus (BVDV Glen Innes) the SNTs against Clover Lane indicated a titre of 1 in 1024 (increased from 1 in 50 observed following vaccination). The titre against Clover Lane rose at 14 days to an average of 1 in 12 000, with one animal giving a titre of 1 in 64 0000 against the sheep isolate. This provides further evidence that the sub-unit vaccine provides wide spread protection against all Australian cattle and sheep pestiviruses. This protection is far greater than presently available with inactivated whole virus vaccines.

Additional SNTs were carried out against 4 Australian pestiviruses, including the unrelated Braidwood BVD virus. Two groups of animals were vaccinated with two different vaccines derived from the Trangie+ Bega+ Clover Lane isolates and were thus directly comparable. In the first group, animals were vaccinated with an experimental inactivated whole virus vaccine. The second group of animals was vaccinated with the sub-unit vaccine (+hsps). Serum was collected from the vaccinated cattle in both groups 4 weeks after 2 doses of the vaccine.

The results for the SNTs against the 3 viruses are shown in Table 14. A comparison of all 3 viruses showed that the sub-unit vaccine resulted in titres at least 4 times higher than the corresponding titres induced by the inactivated vaccine. In addition, cross neutralisation occurred for both vaccines against the totally unrelated BVDV isolate "Braidwood", which showed a similar 4 fold increase in the titre at 4 weeks post vaccination with the sub-unit vaccine when compared to the inactivated vaccine (Table 14).

Thus the requirement for a wider ranging vaccine in all cattle-producing countries is met by the development of this sub-unit vaccine.

Table 14

Animal Number	Reciprocal Serum Neutralisation Titre	
	Inactivated Vaccine (Trangie + Bega)	Sub-unit Vaccine (Trangie + Bega + Clover Lane)
1	32	1024
2	128	64
3	128	128
4	128	256
5	128	256
6	32	16
7	256	512
8	4	1024
9	-	64
10	-	1024
Mean Titre (Range)	104 (4 - 256)	436 (16 - 1024)
± SD	± 75.6	± 407

Comparison of Serum Neutralisation Titre (SNTs) against the “Braidwood” BVDV isolate in serum taken from cattle vaccinated with the CSL inactivated vaccine and the sub-unit vaccine. (Anti-E2 glycoproteins antibodies at 4 weeks after vaccination in both cases).

Effect of sub-unit vaccines on transfer of BVDV to the fetus

Tissue samples collected from foetuses (n=7) obtained from the pregnant vaccinated heifers and from foetuses (n=9) obtained from the pregnant control heifers were tested at EMAI using 3 different BVDV-specific assays (Table 15) as described previously. It was apparent from all 3 tests that there was no BVDV infections in any of the 7 foetuses obtained from the pregnant vaccinated heifers. In contrast, 5 of the 9 foetuses in the control group were infected as shown by the virus isolation and RT-PCR assays.

Therefore, it was concluded that vaccination gave 100% protection against a live, heterologous BVDV challenge at a time when there is a maximum chance of transferring virus into the developing foetus.

Table 15

Foetus harvested from Animal No.	Antigen ELISA (PACE) Results (S/N Ratios) *				Virus Isolation & 5' UTR RT-PCR Results	
	E2✢	NS3	E2 + NS3	Final Result	VI 2nd Pass	RT-PCR (+/-)
Vaccinates						
Q239	1.0	1.1	-ve	-ve	-ve	-ve
Q306	1.0	0.9	-ve	-ve	-ve	-ve
Q324	1.0	1.2	-ve	-ve	-ve	-ve
Q354	1.0	1.1	-ve	-ve	-ve	-ve
Q355	0.9	1.0	-ve	-ve	-ve	-ve
Q379	1.2	1.1	-ve	-ve	-ve	-ve
R346	1.0	0.9	-ve	-ve	-ve	-ve
Controls						
Q240	1.2	1.1	1.0	-ve	-ve	-ve
Q316✦	0.7	0.6	<1.0	-ve	**+ve✻**	**+ve**
Q352	1.0	1.0	1.0	-ve	-ve	-ve
Q373	0.9	1.1	1.0	-ve	-ve	-ve
Q388	**2.9**	**13.6**	**15.8**	**+ve**	**+ve**	**+ve**
R298	**4.9**	**10.8**	**11.1**	**+ve**	**+ve**	**+ve**
Q337	1.0	0.9	1.0	-ve	-ve	-ve
Q372	**3.2**	**12.8**	**14.1**	**+ve**	**+ve**	**+ve**
Q385	**2.3**	**16.2**	**17.6**	**+ve**	**+ve**	**+ve**

.(✻)= signal-to-noise ratios. Ratio >2.0 are positive in PACE on foetal tissues. (✢)= Positive E2 results low. Results confirmed by high S/N ratios with NS3 monoclonals on positive tissues. (✦)= foetus was clearly dead in utero. Results confirmed foetus was infected. (✻) Weak virus isolation positive-only individual cells strained on microplate. Virus titre therefore low in this dead fetus. Results confirmed by diagnostic RT-PCR

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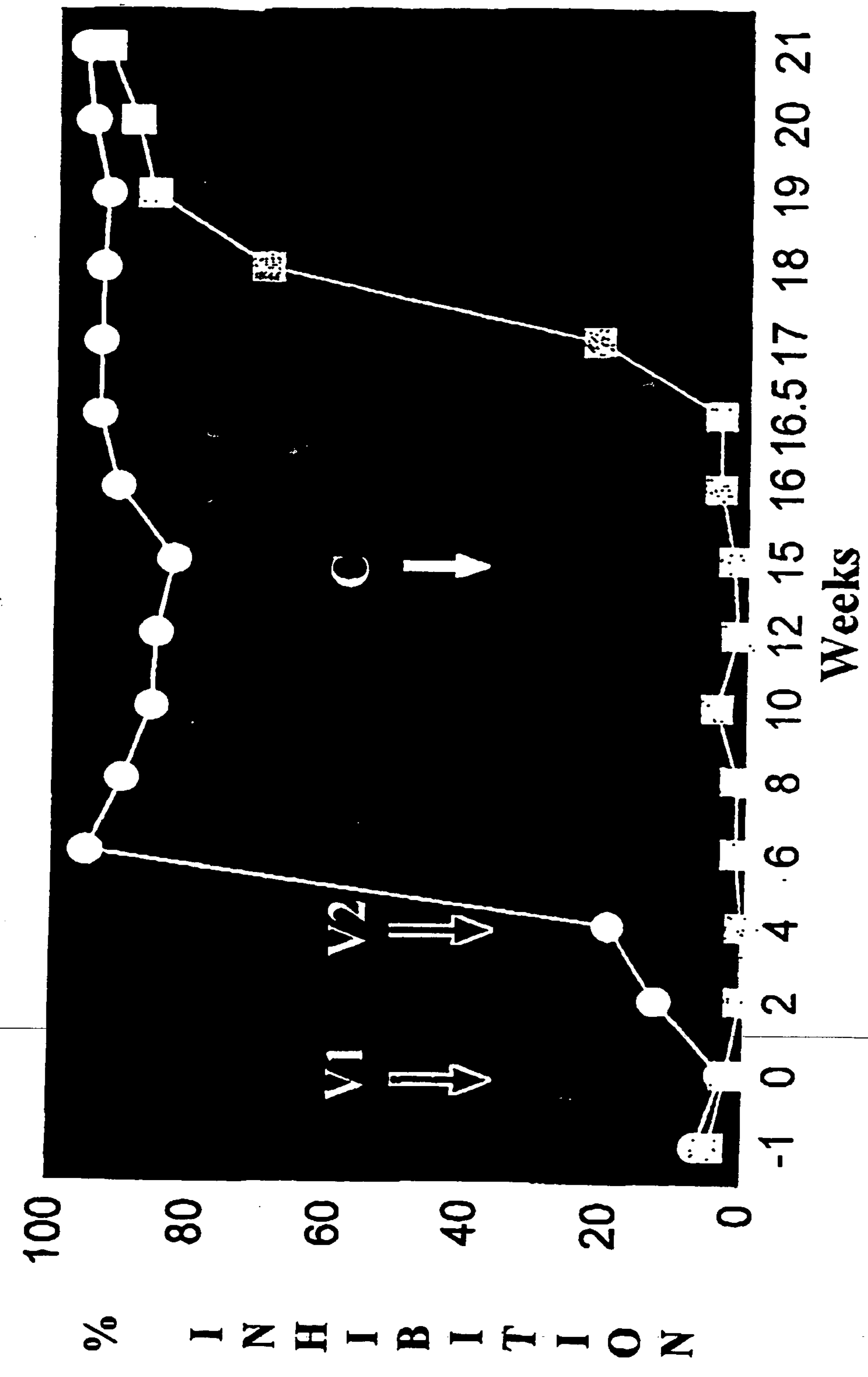
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Fig. 1

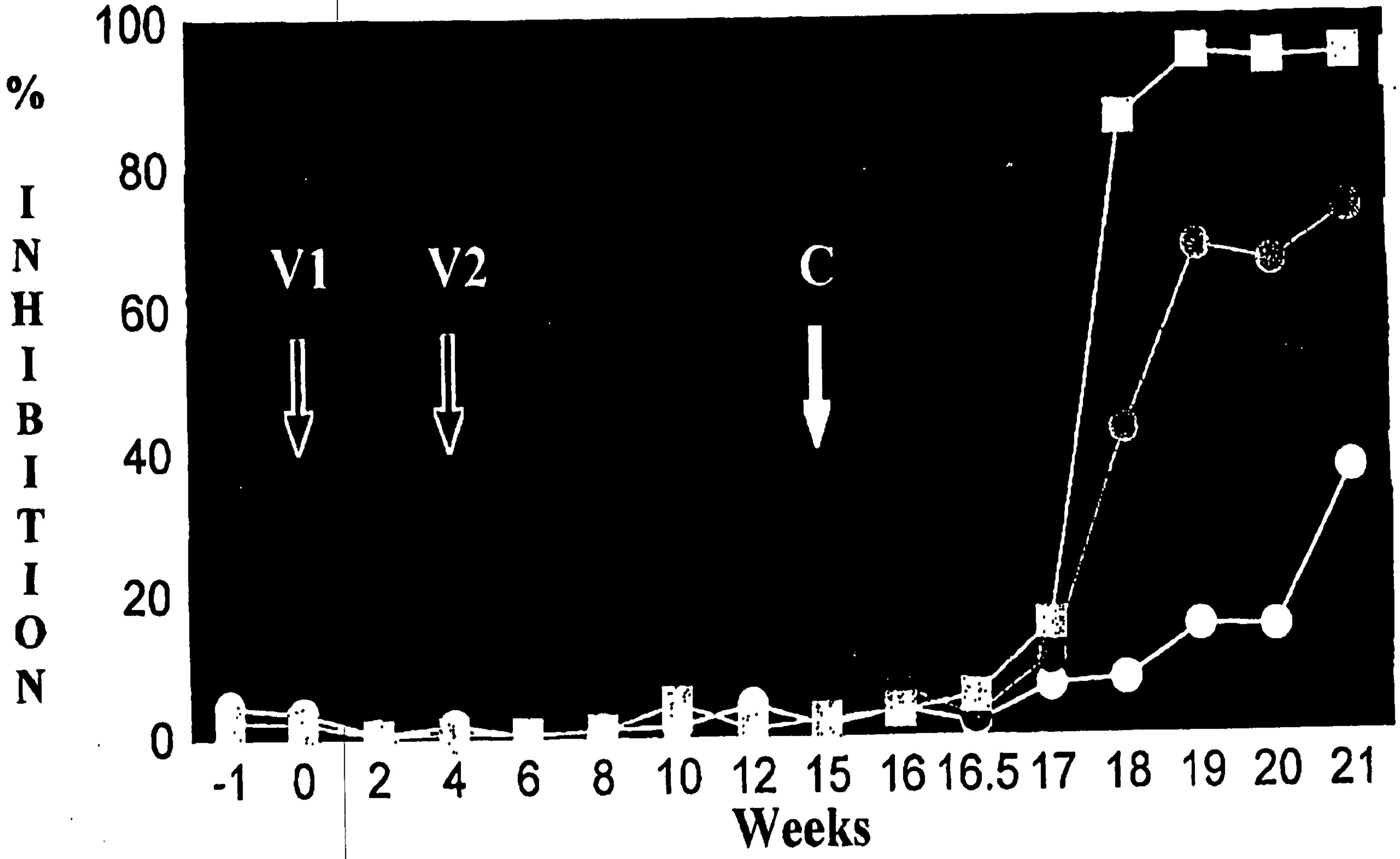
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Fig. 2